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3:	Full name, address and postcode of the or of each applicant *(underline all surnames)*	GlaxoSmithKline Biologicals s.a. Rue de l'Institut 89, B-1330 Rixensart, , Belgium
	Patents ADP number *(if you know it)*	8101271001
	If the applicant is a corporate body, give the country/state of its incorporation	Belgian
4.	Title of the invention	Immunogenic composition
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PHARMACEUTICAL COMPOSITION

The present invention relates to the field of pharmaceutical compositions that can be used in the treatment and prevention of Bordetella infection. In particular, pharmaceutical preparations of the invention will contain a Bordetella protein that is involved in resistance to killing by complement such as BrkA as well as other Bordetella antigen(s) such as pertussis toxin which are involved in resistance of Bordetella to cellular immunity mechanisms.

The bacterium *Bordetella pertussis* is the causative agent for whooping cough, a respiratory disease that can be severe in infants and young children. The clinical course of the disease is characterised by paroxysms of rapid coughs followed by inspiratory effort, often associated with a characteristic 'whooping' sound. In serious cases, oxygen deprivation can lead to brain damage, however the most common complication is secondary pneumonia.

The first generation of vaccines against *B. pertussis* were whole cell vaccines, composed of whole killed bacteria. These were introduced in many countries in the 1950s and 1960s and were successful at reducing the incidence of whooping cough. A problem with whole cell *B. pertussis* vaccines is the high level of reactogenicity associated with them. Acellular vaccines containing purified *B. pertussis* proteins are less reactogenic and have been adopted for the vaccination programmes of many countries. Acellular vaccines typically containing pertussis toxin (PT), filamentous haemagglutinin (FHA) and quite often pertactin (PRN), are widely used and provide effective protection from the severity of whooping cough.

Despite vaccination, whooping cough remains an endemic disease (Mooi et al (2001) Emerging Infectious Diseases 7; 526). Whooping cough has re-emerged in Australia, Canada and The Netherlands; countries with highly vaccinated populations. A comparison of pre-vaccination strains with strains isolated recently, has shown antigenic drift, particularly in PT and PRN (Mooi et al (1998) Infection and Immunity 66; 670). It is widely acknowledged that current vaccines protect against severe disease but do not eliminate *Bordetella pertussis* from the body (Cherry et al (1998) Vaccine 16; 1901, Hewlett and Halperin (1998) Vaccine 16; 1899, Storsaeter et al (1998) Vaccine 16; 1907). The defence mechanisms of *Bordetella pertussis* allow it to evade elimination from the body,

indicating that current vaccines do not completely disable these defence mechanisms. The acellular vaccines currently available elicit antibody that will disrupt the functioning of pertussis toxin but do not elicit a response that would neutralise the capacity of Bordetella to avoid killing by the complement pathway.

Whooping cough is usually considered to be caused by *B. pertussis*, but occasionally *B. parapertussis* is isolated from patients with typical signs and symptoms of whooping cough. *B. parapertussis* infection is of lower frequency than *B. pertussis* with 5-10% of whooping cough being associated with *B. parapertussis* (Mertsola (1985) Eur J Clin Microbiol 4; 123; Lautrop (1971) Lancet 1(7711) 1195-1198). *B. parapertussis* is associated with mild clinical symptoms which, combined with its serological cross-reactivity with *B.pertussis*, makes *B. parapertussis* difficult to diagnose.

Vaccination using whole cell *B. pertussis* vaccines (Pw), appears to protect against *B. parapertussis* infection, probably due to the similarity of the two bacteria. *B. parapertussis* infection in unvaccinated infants may lead to severe and fatal complications, whereas in individuals vaccinated with Pw, a milder, often subclinical course of whooping cough is seen (Long et al (1990) Paediatric Infect Dis J 9; 700). Theoretically, the introduction of acellular pertussis vaccines containing only two or three purified proteins could reduce the ability of vaccination to protect against *B. parapertussis*. Some published studies have failed to show this effect and in fact the studies of Stehr et al have indicated that vaccination with DTPa provides greater protection against *B. parapertussis* than vaccination with DTPw (Stehr et al (1998) Pediatrics 101; 1).

The present invention relates to a acellular Bordetella vaccine that would elicit antibodies to both proteins involved in resistance to killing by complement and proteins involved in evading cellular immunity. Such a vaccine would have the advantage of enabling both phagocytosis and complement fixation to continue to operate against Bordetella, thus eliminating more of the Bordetella defence mechanisms than current acellular vaccines.

An additional advantage of the invention, in aspects where BrkA is included in a vaccine, is that immunity may be generated against *B. parapertussis* as well as *B. pertussis*.

Summary of the invention

In one aspect, the invention relates to pharmaceutical compositions comprising BrkA, FHA and PT. Another aspect of the invention relates to vaccines that elicit an immune response against Bordetella, comprising a protein involved in Bordetella resistance to complement and a protein involved in Bordetella resistance to phagocytosis. A further aspect of the invention is the method of vaccination against Bordetella infection.

Description of figures

Figure 1 – is a graph showing protection against challenge with *B. pertussis* strain Tohama in groups of mice pre-immunised with carrier DT BrkA, DTPa-2, DTPa-2 BrkA, DTPa-3 or DTPa-3 BrkA. Results are expressed as the number of CFU isolated per lung at different time points after challenge.

Figure 2 – is a graph showing protection against challenge with *B. pertussis* strain 18323 in groups of mice pre-immunised with carrier DT BrkA, DTPa-2, DTPa-2 BrkA, DTPa-3 or DTPa-3 BrkA. Results are expressed as the number of CFU isolated per lung at different time points after challenge.

Figure 3 – graphs showing protection against challenge with *B. pertussis* or *B. parapertussis* in groups of mice preimmunised with DTPw or DTPa from several sources. Results are expressed as number of CFU isolated from the lung at different time points after challenge.

Description of the invention

Bordetella pertussis is an obligate human pathogen and has developed mechanisms to survive within the hostile environment of the human host. One mechanism of doing this is through the action of pertussis toxin, which catalyses the ADP-ribosylation of GTP-binding proteins of mammalian cells. Since GTP-binding proteins are signalling molecules involved in regulating cellular processes, such ADP-ribosylation can lead to disruption of cellular function. Several important cells of the immune system including neutrophils, macrophages, monocytes and lymphocytes are inhibited by pertussis toxin (Weiss (1997) ASM News 63; 22). The action of pertussis toxin therefore disables the cellular immune response to *B. pertussis*.

The complement system is another important defence mechanism in the human body. The level of complement in the lung is ordinarily 10-20% of that in serum, however this

increases during inflammation (Persson (1991) Eur. Respir. 4; 1268). *B. pertussis* has developed mechanisms of evading the complement system. Firstly, the lipopolysaccharides of *B. pertussis* do not activate the alternative pathway of complement (Fernandez and Weiss (1994) Infection and Immunity 62; 4727). The binding of antibodies to *B. pertussis* could however, lead to activation of the classical complement pathway. *B. pertussis* has developed a mechanism of inhibiting the classical complement pathway, using the protein BrkA.

BrkA is expressed in *B. pertussis, B. parapertussis* and *B. bronchiseptica* and falls into the family of autotransporter proteins (Fernandez and Weiss (1998) FEMS Microbiol Lett 163; 57). The DNA and amino acid sequence of BrkA is published by Fernandez and Weiss (Infection and Immunity Nov 1994, 62; 4727-4738) and can be accessed from the NCBI Entrez nucleotide database under accession number U12276. The 103kDa BrkA is proteolytically processed into a 73kDa N-terminal domain and a 30kDa C-terminal domain (Henderson and Nataro (2001) Infection and Immunity 69; 1231). Translocation of the N-terminal domain across the outer membrane is hypothesised to occur through a pore formed by the C-terminal domain. It has been demonstrated that the C-terminal domain of BrkA is capable of forming such a pore (Shannon and Fernandez (1999) J. Bacteriology 181; 5838).

The principle function of BrkA is as an adhesin/invasin, allowing *B. pertussis* to bind to host cells, probably through its RGD sequences. However BrkA has an additional function in mediating serum resistance (Fernandez and Weiss (1998) FEMS Microbiol. Lett. 163; 57). The mechanism by which BrkA inhibits the classical complement pathway is not completely understood. However, it appears to prevent the accumulation of deposited C4, suggesting that it acts early in the pathway, before C4 deposition (Barnes and Weiss (2001) Infection and Immunity 69; 3067).

The present invention provides the means to develop an acellular vaccine combining low reactogenicity, with a greater likelihood of eliminating Bordetella from the subject. This is achieved due to two of Bordetella's protection mechanisms being attacked rather than just one, as in conventional Bordetella vaccines. Thus the mechanism by which Bordetella evades killing by complement could be targeted as well as the mechanism by which Bordetella evades cellular immunity, currently targeted by eliciting an immune response against PT. Such a vaccine would have the advantage of eliminating more Bordetella from

the host, decreasing the pool from which infection could result and decreasing the chance of antigenic drift.

An additional advantage of the invention, in aspects in which BrkA is included in a vaccine, is that antibodies may be generated against *B. parapertussis* as well as *B. pertussis*. Contrary to published reports (Stehr et al (1998) Pediatrics 101; 1) we have found that vaccination with DTPa gives less protection against *B. parapertussis* than vaccination with DTPw (Example 6). This result indicates that the change from use of DTPw to DTPa as the preferred vaccine to prevent whooping cough, may lead to a reduced level of protection against *B. parapertussis*. BrkA is conserved between different strains of Bordetella and could contribute to cross-protective antibody production, when included in a vaccine. The addition of other antigens which are present in several Bordetella species (cross-reactive Bordetella antigens) could add to the cross-protective effect. The compositions of the invention may thus fulfil an unmet need of providing an acellular vaccine effective against *B. pertussis* and *B. parapertussis*.

The present invention relates to a pharmaceutical composition, preferably an immunogenic composition, more preferably a vaccine and more preferably an acellular vaccine against Bordetella infection, comprising an antigen involved in Bordetella resistance to complement and an antigen involved in Bordetella resistance to cellular immunity. Such antigens may be proteins, lipoproteins, polysaccharides, lipopolysaccharides or any other constituent of Bordetella.

In a preferred embodiment of the invention, the pharmaceutical composition comprises BrkA and/or BrkB as a protein involved in Bordetella resistance to complement and PT or adenylate cyclase as a protein involved in Bordetella resistance to cellular immunity. Lipopolysaccharides (LPS) are antigens that are also toxic to cells involved in immunity and in some embodiments of the invention could supplement or replace pertussis toxin or adenylate cyclase. In a further embodiment of the invention, the pharmaceutical composition comprises BrkA as a protein involved in Bordetella resistance to complement, PT as a protein involved in Bordetella resistance to cellular immunity and FHA. In a further embodiment of the invention, the pharmaceutical composition comprises BrkA as a protein involved in Bordetella resistance to complement, PT as a protein involved in Bordetella resistance to cellular immunity, FHA and 69kDa pertactin.

The pharmaceutical compositions of the invention preferably comprise one or more additional Bordetella antigens which are cross-protective. It is advantageous for a vaccine to generate protection against *B. parapertussis* as well as *B. pertussis* so that a single vaccine can protect against both forms of infection. BrkA is well conserved between *B. pertussis* and *B. parapertussis* and its inclusion in pharmaceutical compositions of the invention allows antibodies to be generated against both strains. However, a better level of protection is achieved by the inclusion of one or more additional antigens which are conserved between the several strains of Bordetella.

Cross-reactive Bordetella antigens are expressed in at least two Bordetella species (preferably including two or more of *B. pertussis, B. parapertussis, B. bronchoseptica*) and include Bordetella adenylate cyclase (AC-Hly), filamentous haemagglutinin (FHA), fimbrial adhesins, pertactin, vag8, vir90, ExbB, ExbD, TonB, BvgS, LT, tracheal CF (tracheal cytotoxin), LPS mimotopes and LPS conjugates. Several methods can be used to identify Bordetella cross-reactive antigens. Using genome mining, a comparison of the genomes of *B. pertussis* and *B. parapertussis* would show which antigens are conserved between the two species. Alternatively, DNA chips could be used alongside sequence information to assess the expression of candidate antigens in *B. pertussis* and *B. parapertussis.* Antisera against Pw could be used to identify cross-reactive antigens by using gel electrophoresis and western blotting. Spot microsequencing could precisely identify cross-reactive antigens. The invention embodies vaccines containing cross-reactive Bordetella antigens identified by the above methods or similar methods.

The individual pertussis components may be purified from native *B. pertussis* using purification procedures which are known to the skilled artisan. Alternatively, the components are obtained using recombinant DNA techniques to express the components. In this case, the antigens are expressed in suitable host cells such as members of the genus Bordetella, *E. coli*, Haemophillus, Streptomyces, *Bacillus subtilis*, yeast, insect cell lines or mammalian cell lines, or any other suitable host expression system.

Purification of the antigens is preferably achieved by affinity chromatography using epitope tags such as his-tags, FLAG-tags, myc-tags or any other amino acid sequence capable of being used in a similar way. Antigens are purified using one or more of affinity chromatography, ion exchange chromatography, gel filtration chromatography, reverse phase chromatography or any other method used to separate antigens and the genes encoded are preferably derived from a pertussis strain.

The PT component is detoxified, preferably using a chemical detoxification method as described in the literature (Munoz et al (1989) Infection and Immunity, 33; 820; Relyveld et al (1987) Methods in Enzymology 93; 24-60; Kaslow et al (1987) Biochemistry 26; 4397) involving treatment with formaldehyde, hydrogen peroxide, tetranitro-methane or glutaraldehyde. Alternatively, PT is detoxified genetically through the introduction of mutations that remove the undesired biological activities of the toxin (Zealey et al (1989) Vaccines 89; 259 and Pizza et al (1989) Science 246; 497).

Preferred ratios of antigens for inclusion into a pharmaceutical composition are 1-10 PT to 1 BrkA or BrkB, more preferred ratios are 1-5 PT to 1 BrkA or BrkB, most preferred ratios are 2.5 PT to 1 BrkA or BrkB (w/w).

The pertussis antigens may be unconjugated or conjugated to bacterial structural components, preferably capsular polysaccharides or oligosaccharides using conventional techniques. Components may be directly conjugated by reductive animation (US4, 673, 574), or may be linked through a spacer element such as adipic acid dihydrazide or 6-aminocaproic acid (US4,459.286).

It is advantageous for pharmaceutical compositions of the invention to further comprise immunogenic amounts of additional antigens to elicit immunity to other pathogens. Such additional antigens include diphtheria toxoid, tetanus toxoid, hepatitis B surface antigen, inactivated polio virus (IPV), *Haemophilus influenzae* type b PRP polysaccharide or oligosaccharide, fimbria 2 and fimbria 3. Typically, the antigens providing protection against Diphtheria and tetanus would be Diphtheria toxoid and tetanus toxoid. The toxoids may chemically inactivated toxins or toxins inactivated by the introduction of point mutations. Preferred additional component are both tetanus toxoid and diphtheria toxoid, more preferably Tetanus toxoid, diphtheria toxoid and IPV, more preferably tetanus toxoid, diphtheria toxoid, IPV and Hepatitis B surface antigen, most preferably tetanus toxoid, diphtheria toxoid, IPV, Hepatitis B surface antigen and Haemophilus influenzae b polysaccharide.

A further aspect of the invention are vaccine combinations comprising the antigenic composition of the invention with other antigens which are advantageously used against certain disease states including those associated with viral or Gram positive bacteria.

In a further preferred embodiment, the antigenic compositions of the invention are formulated with a conjugated *H. influenzae* b capsular polysaccharide or oligosaccharide, and one or more plain or conjugated pneumococcal capsular polysaccharides or oligosaccharides. Optionally, the vaccine may also comprise one or more protein antigens that can protect a host against *Streptococcus pneumoniae* infection. Such a vaccine may be advantageously used as a Bordetella/H. influenzae/streptococcus pneumonia vaccine. A further preferred embodiment would comprise capsular polysaccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F).

Pneumococcal protein antigens could be added to pharmaceutical compositions of the invention. Preferred pneumococcal proteins are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal transducer, or lipoprotein of *Streptococcus pneumoniae*, or fragments thereof. Particularly preferred proteins include, but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2."; Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC and transmembrane deletion variants thereof (WO 97/09994 - Briles et al); PsaA and transmembrane deletion variants thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); pneumococcal choline binding proteins and transmembrane deletion variants thereof; CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP70 (WO 96/40928); PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14); M like protein, (EP 0837130) and adhesin 18627, (EP 0834568). Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390.

In a further preferred combination, the pharmaceutical compositions of the invention are formulated with 1, 2, 3 or preferably all 4 of the following meningococcal capsular polysaccharides or oligosaccharides : A, C, Y or W, which may be plain or conjugated to a protein carrier. Such a vaccine containing proteins from *N. meningitidis* serogroup B may be advantageously combine a global meningococcus vaccine with a Bordetella vaccine.

Capsular polysaccharides or oligosaccharides included in pharmaceutical compositions of the invention may be unconjugated or conjugated to a carrier protein such as tetanus toxoid, tetanus toxoid fragment C, diphtheria toxoid, CRM197, pneumolysin, Protein D (US6342224).

The polysaccharide or oligosaccharide conjugate may be prepared by any known coupling technique. For example the polysaccharide can be coupled via a thioether linkage. This conjugation method relies on activation of the polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may thus be coupled directly or via a spacer group to an amino group on the carrier protein. Preferably, the cyanate ester is coupled with hexane diamine and the amino-derivatised polysaccharide is conjugated to the carrier protein using heteroligation chemistry involving the formation of the thioether linkage. Such conjugates are described in PCT published application WO93/15760 Uniformed Services University. The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide activated polysaccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256).

Vaccine Formulations

Preferably, the pharmaceutical preparations of the invention are immunogenic compositions that are capable of eliciting an immune response against the antigens included in the pharmaceutical composition. A preferred embodiment of the invention is the formulation of the immunogenic composition of the invention in a vaccine which may also comprise a pharmaceutically acceptable excipient or carrier. A vaccine is capable of eliciting a protective immune response against an infectious agent, preferably Bordetella

pertussis, more preferably against Bordetella pertussis and Bordetella parapertussis, and may be used for treatment or prevention of infection.

A vaccine of the invention elicits a protective immune response against Bordetella and comprises an isolated protein involved in Bordetella resistance to complement (or serum resistance), an isolated protein involved in Bordetella resistance to cellular immunity and a pharmaceutically acceptable carrier. A protein involved in Bordetella resistance to complement is defined as a Bordetella protein that is capable of disrupting the effective functioning of the host's complement system preferably by inhibiting the classical complement activation pathway. The degree of inhibition will be at least 10%, preferably 20%, more preferably 30%, more preferably 40%, 50%, 60%, 70%, 80%, most preferably 90% or 95%. This may be measured by the ability of the protein to inhibit a serum killing assay as described in Infect. Immun. 69; 3067 (2001). Examples of this sort of protein include BrkA and BrkB from Bordetella and fragmetns thereof eliciting an immunogenic response against said protein, in particular the passenger domain (approximately from amino acid 41 to amino acid 706). A protein involved in Bordetella resistance to cellular immunity is defined as a Bordetella protein which is able to inhibit the effective functioning of at least one type of cell making up the host's cellular immunity system. It may act by having a toxic effect on one or more of the host's cell populations involved in cellular immunity, for instance T lymphocytes, B lymphocytes, neutrophils, eosinophils, macrophages, dendritic cells or monocytes. Examples of such antigens include pertussis toxin, adenylate cyclase and LPS. It may alternatively inhibit cellular immunity by disrupting the function of cell involved in immunity. It is envisaged that non-toxic derivatives of such proteins may also be used that are immunologically similar to the native protein e.g. toxoids or non-toxic mutants.

Vaccines of the invention comprise all the pharmaceutical compositions of the invention described above, combined with a pharmaceutically acceptable carrier. Vaccines of the invention elicit an effective immune response against *Bordetella pertussis* and preferably against *Bordetella pertussis* and *Bordetella parapertussis.*

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

The pharmaceutical compositions of the present invention may be adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble

suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be used include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

The vaccine may comprise a saponin, more preferably QS21. It may also comprise an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Thus one aspect of the present invention is a method of immunizing a human host against a disease caused by Bordetella infection, which method comprises administering to the host an immunoprotective dose of the pharmaceutical preparation of the present invention.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of protein antigen, preferably 5-50μg, and most typically in the range 5 - 25μg.

An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

The vaccine of the invention may be used to treat or prevent Bordetella infection in adults or in paediatrics. By paediatric use it is meant use in infants less than 4 years old.

By immunoprotective it is meant that at least 40% (and preferably 50, 60, 70, 80, 90 and 100%) of infants seroconvert (4-fold increase in bactericidal activity [the dilution of antisera at which 50% of bacteria die – see for example PCT/EP98/05117]) against a set of heterologous strains to be selected from the major clonal groups known.

By non-toxic it is meant that there is a significant (2-4 fold, preferably 10 fold) decrease of endotoxin activity as measured by the well-known LAL and pyrogenicity assays.

Suitable preservatives such as 2-phenoxyethanol, thimersol, dextran and glycerine can be added to stabilise the vaccine.
A further aspect of the invention is a method of eliciting a protective immune response against Bordetella , preferably Bordetella pertussis or B. parapertussis, more preferably B. pertussis and B. parapertussis by administering the vaccine of the invention. Where combination vaccines of the invention are administered, methods of the invention comprise eliciting a protective immune response against one or more infections selected from the group consisting of *Bordetella,* diphtheria, tetanus, polio, hepatitis and *Haemophilus influenzae* type b, Streptococcus pneumoniae and Neisseria meningitidis. Methods of the invention also include methods of preventing or treating infection by one or more infectious agent selected from the group consisting of *Bordetella,* diphtheria, tetanus, polio, hepatitis, *Haemophilus influenzae* type b, Streptococcus pneumoniae and Neisseria meningitidis.

A further aspect of the invention is the use of the pharmaceutical composition or vaccine of the invention in the manufacture of a medicament for the treatment or prevention of one or more infections selected from the group consisting of, Bordetella, Bordetella *pertussis,* diphtheria, tetanus, polio, hepatitis, *Haemophilus influenzae* type b, Streptococcus pneumoniae and Neisseria.

Antibodies and passive immunisation

Another aspect of the invention is the use of an immunogenic composition or vaccine comprising an isolated protein involved in Bordetella resistance to complement and an isolated protein involved in Bordetella resistance to cellular immunity to generate immune globulin which can be used to treat or prevent Bordetella infection, preferably *Bordetella pertussis* infection more preferably *Bordetella parapertussis* infection most preferably

both. Preferably, the immunogenic composition or vaccine comprises BrkA, more preferably it comprises BrkA and PT, most preferably it comprises BrkA, and an additional Bordetella antigen, for example FHA, pertactin, adenylate cyclase, vag8, vir90, ExbD, ExbD, TonB, BvgS, LT, Tracheal CF, LPS mimotopes or LPS conjugates.

Inocula for polyclonal antibody production are typically prepared by dispersing the antigenic composition in a physiologically tolerable diluent such as saline or other adjuvants suitable for human use to form an aqueous composition. An immunostimulatory amount of inoculum is administered to a mammal and the inoculated mammal is then maintained for a time sufficient for the antigenic composition to induce protective antibodies.

The antibodies can be isolated to the extent desired by well known techniques such as affinity chromatography.

Antibodies can include antiserum preparations from a variety of commonly used animals e.g. goats, primates, donkeys, swine, horses, guinea pigs, rats or man. The animals are bled and serum recovered.

An immune globulin produced in accordance with the present invention can include whole antibodies, antibody fragments or subfragments. Antibodies can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments. An immune globulin also includes natural, synthetic or genetically engineered proteins that acts like an antibody be binding to specific antigens to form a complex.

A vaccine of the present invention can be administered to a recipient who then acts as a source of immune globulin, produced in response to challenge from the specific vaccine. A subject thus treated would donate plasma from which hyperimmune globulin would be obtained via conventional plasma fractionation methodology. The hyperimmune globulin would be administered to another subject in order to impart resistance against or treat Bordetella infection. Hyperimmune globulins of the invention are particularly useful for treatment or prevention of Bordetella disease in infants, immune compromised individuals or where treatment is required and there is no time for the individual to produce antibodies in response to vaccination.

An additional aspect of the invention is a pharmaceutical composition comprising monoclonal antibodies reactive against BrkA which could be used to treat or prevent infection by Bordetella or preferably *Bordetella pertussis*, more preferably *Bordetella parapertussis* most preferably both. Monoclonal antibodies against BrkA would be combined with monoclonal or polyclonal antibodies against other Bordetella antigens so that protein involved in Bordetella resistance to complement killing and proteins involved in Bordetella resistance to cellular immunity would be bound by the combination of antibodies.

Such pharmaceutical compositions comprise monoclonal antibodies that can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity to Tbp and Hsf. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments.

Methods of making monoclonal antibodies are well known in the art and can include the fusion of splenocytes with myeloma cells (Kohler and Milstein 1975 Nature 256; 495; Antibodies – a laboratory manual Harlow and Lane 1988). Alternatively, monoclonal Fv fragments can be obtained by screening a suitable phage display library (Vaughan TJ et al 1998 Nature Biotechnology 16; 535).

A further embodiment of the invention is a method of preventing and treating Bordetella infection comprising administration of immune globulin obtained from a mammal immunised with a vaccine or pharmaceutical composition of the invention. The method is preferably used to treat or prevent Bordetella infection in immuno-compromised individuals or neonates.

A further embodiment of the invention is a use of an immune globulin obtained by administering a vaccine of the invention in the manufacture of a medicament for preventing or treating Bordetella infection, preferably for prevention or treatment of Bordetella infection in immuno-compromised individuals or neonates.

Examples

Example 1 Expression of BrkA

BrkA is a 1010 amino acid protein. A pET30b expression vector containing amino acids 60-702 of BrkA, fused at the C and N-terminal to a 6x-His tag was used to express BrkA in *E. coli*. The bacteria were grown to an OD600 of approximately 0.6 and induced with 1mM IPTG for 2 hours. Recombinant BrkA was purified under denaturing conditions using the protocol in the Xpress System Protein Purification manual (Invitrogen, Carlsbad, CA). The bacteria were lysed in 6M guanidine hydrochloride and the lysate was applied to to Ni2+ -nitrilotriacetic acid agarose (Qiagen, Mississauga, Ont.). After successive washes in 8M urea of decreasing pH, purified BrkA was eluted at pH 4 and the fractions were pooled. The urea was removed by slow dialysis at 4 °C against 10mM Tris, pH 8.0 in the presence of 0.1% Triton X-100.

The purified protein was refolded so that it has a beta-structure resembling PRN (monitored by CD) and was functional in a serum assay. The protein was filter sterilized and is diluted in 10mM Tris buffer, pH 8 at a concentration of approximately 0.4mg/ml.

Example 2. Formulation of vaccines

Six vaccines were formulated:

DTPa018A2 – contains 6.25Lf DT, 2.5Lf TT, 6.25ug PT and 6.25ug FHA per mouse dose. All antigens were adsorbed separately onto Al(OH)3 before combining.

DTPa14885B9 - contains 6.25Lf DT, 2.5Lf TT, 6.25ug PT, 6.25ug FHA and 2ug pertactin per mouse dose. . All antigens were adsorbed separately onto Al(OH)3 before combining.

DTPw13126A9 – contains 6.25Lf DT, 2.5Lf TT and 1 I.U.whole cell *B. pertussis* per mouse dose. All antigens were adsorbed separately onto Al(OH)3 before combining.

DTBrkA - contains 6.25Lf DT, 2.5Lf TT and 2.5ug BrkA per mouse dose. All antigens were adsorbed separately onto Al(OH)3 before combining.

DTPa-2 BrkA - contains 6.25Lf DT, 2.5Lf TT, 6.25ug PT, 6.25ug FHA and 2.5ug BrkA per mouse dose. . All antigens were adsorbed separately onto Al(OH)3 before combining.

DTPa-3 BrkA - contains 6.25Lf DT, 2.5Lf TT, 6.25ug PT, 6.25ug FHA, 2ug pertactin and 2.5ug BrkA per mouse dose. All antigens were adsorbed separately onto Al(OH)3 before combining.

Example 3 Protection against lung invasion by *B. pertussis* in an animal model

Groups of 20 BALB/c mice (females, 5 weeks old) were immunized subcutaneously with ¼ of a human dose (125 µl of vaccine) and were boosted 3 weeks later. One week after the booster, a sample of blood was collected from each mouse for antibody determination. The mice were then challenged by instillation of 50 µl of bacterial suspension (+/- 5 10^6 CFU/50 µl) into the left nostril under ether anesthesia. Five mice in each group were killed at 4 different times (2 hours, 2, 5 and 8 days) after challenge and the lungs were removed

aseptically and homogenized individually. The log10 weighted mean number of CFU/lung was determined by counting the colonies grown on Bordet-Gengou agar plates after plating of 100 µl of 4 serial dilutions of the homogenate. The arithmetic mean of the log10 weighted mean number of CFU/lung and the standard deviation were calculated for each group and each time point.
Three experiments were performed. The same vaccine groups were included in each experiment but animals were either challenged with *B. pertussis* strain Tohama, *B. pertussis* strain 18323 or *B. parapertussis*.
The day before challenge, blood was collected from each mouse. The anti-PT, anti-FHA, anti-PRN and anti-BrkA antibody levels were determined by ELISA. The geometrc mean titre for each group of mice was calculated.

<u>Example 4 *B. pertussis* strain Tohama challenge</u>

In one experiment immunized mice were challenged with *B. pertussis* strain Tohama. The number of CFU/lung at each timepoint after challenge and for each group is summarized in the figure 1. The experiment had low variability with the data having a mean square error of is 0.450.
Statistical analysis of the data using ANOVA1 was used to assess the data. No significant difference was seen beween the protection against *B. pertussis* offered by DTBrkA conpared to control, indicating that immunisation with BrkA alone is insufficient to elicit protection. In contrast, the addition of BrkA to a DTPa-2 vaccine produced a statistically significant increase in protection showing that, in combination with PT and FHA, BrkA can produce additional protection. The level of protection conferred by DTPa-2 BrkA was statistically slightly less than that conferred by DTPa-3 which conferred protection statistically equivalent to DTPw. The DTPa-3 BrkA vaccine provided excellent protection from challenge after 2 and 5 days but less protection after day 8.

<u>Example 5 *B. pertussis* strain 18323 challenge</u>

In this experiment, immunized mice were challenged with *B. pertussis* strain 18323. The number of CFU/lung at each timepoint after challenge and for each group is summarized in figure 2. The experiment showed low variability with the mean square error of the experiment being 0.402. Statistical analysis using ANOVA1 showed that again DTBrkA did not provide an significant protection over the control. However, DTPa-2 BrkA provided better protection than DTPa, showing that BrkA, in combination with other *B. pertussis* antigens gives additional protection. The protection achieved by vaccination with DTPa-2 BrkA|, against challenge with *B. pertussis* strain 18323, was statistically equivalent to that provided by DTPa-3, DTPa-3 BrkA and DTPw.

<u>Example 6 Comparison of protection against *B. pertussis* and *B. parapertussis* in mice vaccinated with DTPw or DTPa</u>

Groups of 25 or 30 BALB/c mice (females, 5 weeks old) were immunized subcutaneously with ¼ of a human dose of DT, DTPa or DTPw from different sources (125 µl of vaccine) and were boosted 3 weeks later. The sources of DTPw were Triple antigen (CSL), Tri-immune (Lederle), Pentacoq (MSD), Combivax (Behring), Infanrix (SB), DKTP (RVM), DTPw (Connaght) and Trivax (Wellcome). The sources of DTPa were Infanrix (SB), Triacel (PMCS), DI-TE-KIK (Amvax), Acell-immune (Lederle), Tropedia (Biken), Tricelluvax (Biocine/Chiron), Pentavac (PM-MSD) and DTPa-2 (SB). One or two weeks after the booster, a sample of blood was collected from each mouse for antibody determination. The mice were then challenged by instillation of 50 µl of bacterial suspension (+/- 5 10^6 CFU/50 µl) into the left nostril under anaesthesia. Five mice in each group were killed at 5 or 6 different times (ranging from 2 hours to 14 days) after challenge and the lungs were removed aseptically and homogenized individually. The log10 weighted mean number of CFU/lung was determined by counting the colonies grown on Bordet-Gengou agar plates after plating of 100 µl of 4 serial dilutions of the homogenate. The arithmetic mean of the log10 weighted mean number of CFU/lung and the standard deviation were calculated for each group and each time point.
The day before challenge, blood was collected from each mouse for determination of the anti-PT, anti-FHA and anti-PRN antibody levels by ELISA. The geomean titre for each group of mice was calculated.
Results were analysed statistically by applying 1-way and 2-way ANOVA after assuming equality of variance (checked by Brown and Forsythe's test) and normality (checked using the Shapiro-Wilk test). Differences between groups were analysed using the Dunnet test, Tukey's studentised range test (HSD) and Student-Newman-Keuls test.

Results are shown in figure 3, and show that the DTPw vaccines induced good lung clearance of *B. pertussis* and *B. parapertussis*. However, DTPa vaccines induced strong lung clearance of *B. pertussis* but only limited clearance of *B. parapertussis*.

This experiment demonstrates that antigens present in the DTPw vaccine and absent from the DTPa vaccine are protective against parapertussis. Moreover, the antigens present in the DTPa vaccine, specifically FHA and pertactin, may display antigenic variability when compared to the corresponding parapertussis antigens.

CLAIMS

1. A pharmaceutical composition comprising Bordetella BrkA, FHA and PT and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition according to claim 1 further comprising Bordetella pertactin .

3. The pharmaceutical composition according to claim 1 or 2 further comprising an additional, cross-reactive, Bordetella antigen.

4. The pharmaceutical composition according to claim 1 or 2 further comprising at least one additional Bordetella antigen selected from the group containing adenylate cyclase, vag8, vir90, ExbB, ExbD, TonB, BvgS, LT, Tracheal CF, LPS mimotopes and LPS conjugates.

5. The pharmaceutical composition according to any preceding claim further comprising diphtheria toxoid and tetanus toxoid.

6. The pharmaceutical composition according to any preceding claim further comprising hepatitis B surface antigen.

7. The pharmaceutical composition according to any preceding claim further comprising inactivated polio virus (IPV).

8. The pharmaceutical composition according to any preceding claim further comprising *Haemophilus influenzae* type b PRP capsular polysaccharide or oligosaccharide, or a protein conjugate thereof.

9. The pharmaceutical composition according to any preceding claim further comprising fimbria 2 and fimbria 3.

10. The pharmaceutical composition according to any preceding claim further comprising an immunogenic amount of a *Streptococcus pneumoniae* capsular polysaccharide or oligosaccharide, or a protein conjugate thereof.

11. The pharmaceutical composition according to any preceding claim further comprising an immunogenic amount of at least one of the following meningocaccal polysaccharides or oligosaccharides, or protein conjugate thereof: Men A, Men C, Men Y or Men W antigens.

12. The pharmaceutical composition of claim 1-11 that elicits an immune response against *B. pertussis*.

13. The pharmaceutical composition of claim 1-12 that elicits an immune response against *B. parapertussis*.

14. A vaccine that elicits an immune response against Bordetella comprising:
a) an isolated protein involved in *Bordetella* resistance to complement;
b) an isolated protein involved in *Bordetella* resistance to cellular immunity; and
c) a pharmaceutically acceptable carrier.

15. The vaccine according to claim 14 comprising an effective amount of BrkA and PT.

16. The vaccine according to claim 14 or 15 that elicits an immune response against *Bordetella pertussis*.

17. The vaccine according to claim 14-16 that elicits an immune response against *Bordetella parapertussis*.

18. The vaccine according to claim 14-17 further comprising an additional, cross-reactive, Bordetella antigen.

19. The vaccine according to claim 14-18 further comprising at least one additional Bordetella antigen selected from the group containing adenylate cyclase, vag8, vir90, ExbB, ExbD, TonB, BvgS, LT, Tracheal CF, LPS mimotopes and LPS conjugates.

20. The vaccine according to claim 14-19 further comprising an immunogenic amount of FHA.

21. The vaccine according to claim 14-20 further comprising an immunogenic amount of pertactin.

22. The vaccine according to claim 14 -21 further comprising an immunogenic amount of diphtheria toxoid and tetanus toxoid.

23. The vaccine according to claim 14-22 further comprising an immunogenic amount of hepatitis B surface antigen.

24. The vaccine according to claim 14-23 further comprising an immunogenic amount of IPV.

25. The vaccine according to claim 14-24 further comprising an immunogenic amount of *Haemophilus influenzae* type b PRP capsular polysaccharide or oligosaccharide or protein conjugate thereof.

26. The vaccine according to claim 14-25 further comprising an immunogenic amount of fimbria 2 and fimbria 3.

27. The vaccine according to claim 14-26 further comprising an immunogenic amount of *Streptococcus pneumoniae* capsular polysaccharide or oligosaccharide or protein conjugate thereof.

28. The vaccine according to claim 14-27 further comprising an immunogenic amount of at least one of the following meningococcal capsular polysaccharide or oligosaccharide or protein conjugate thereof: Men A, Men C, Men Y or Men W antigens.

29. A method of eliciting a protective immune response against Bordetella by administering the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13.

30. A method of eliciting a protective immune response against one or more infections selected from the group containing *Bordetella pertussis,* diphtheria, tetanus, polio, hepatitis and *Haemophilus influenzae* type b by administering the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13.

31. A method of eliciting a protective immune response against *Bordetella pertussis* by administering the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13.

32. Use of the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for the prevention or treatment of Bordetella infection.

33. Use of the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for the prevention or treatment of one or more infections selected from the group containing *Bordetella pertussis,* diphtheria, tetanus, polio, hepatitis and *Haemophilus influenzae* type b.

34. Use of the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for the prevention or treatment of *Bordetella pertussis* infection.

35. A method of preventing and treating Bordetella infection comprising administration of immune globulin obtained from a mammal immunised with the vaccine of claims 14-28 or the pharmaceutical composition of claims 1-13.

36. The method of claim 35 in which the immune globulin is administered to immuno-compromised individuals.

37. The method of claim 35 in which the immune globulin is administered to neonates.

38. The use of an immune globulin obtained by administering a vaccine according to claim 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for preventing or treating Bordetella infection.

39. The use of an immune globulin obtained by administering a vaccine according to claim 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for preventing or treating Bordetella infection in immuno-compromised individuals.

40. The use of an immune globulin obtained by administering a vaccine according to claim 14-28 or the pharmaceutical composition of claims 1-13 in the manufacture of a medicament for preventing or treating Bordetella infection in neonates.

Figure 1

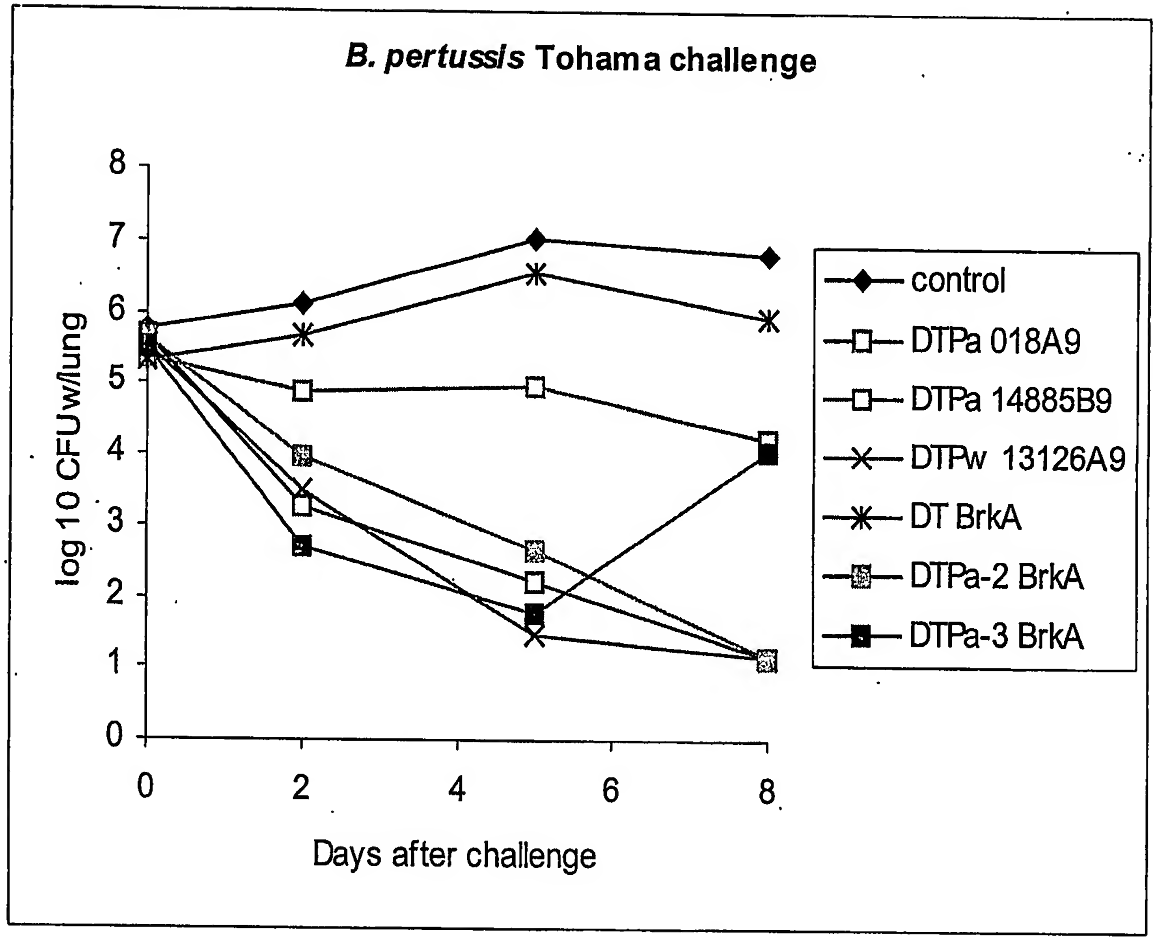

B. pertussis Tohama challenge
log 10 CFUw/lung
Days after challenge
0
1
2
3
4
5
6
7
8
0
2
4
6
8
control
DTPa 018A9
DTPa 14885B9
DTPw 13126A9
DT BrkA
DTPa-2 BrkA
DTPa-3 BrkA

Figure 2

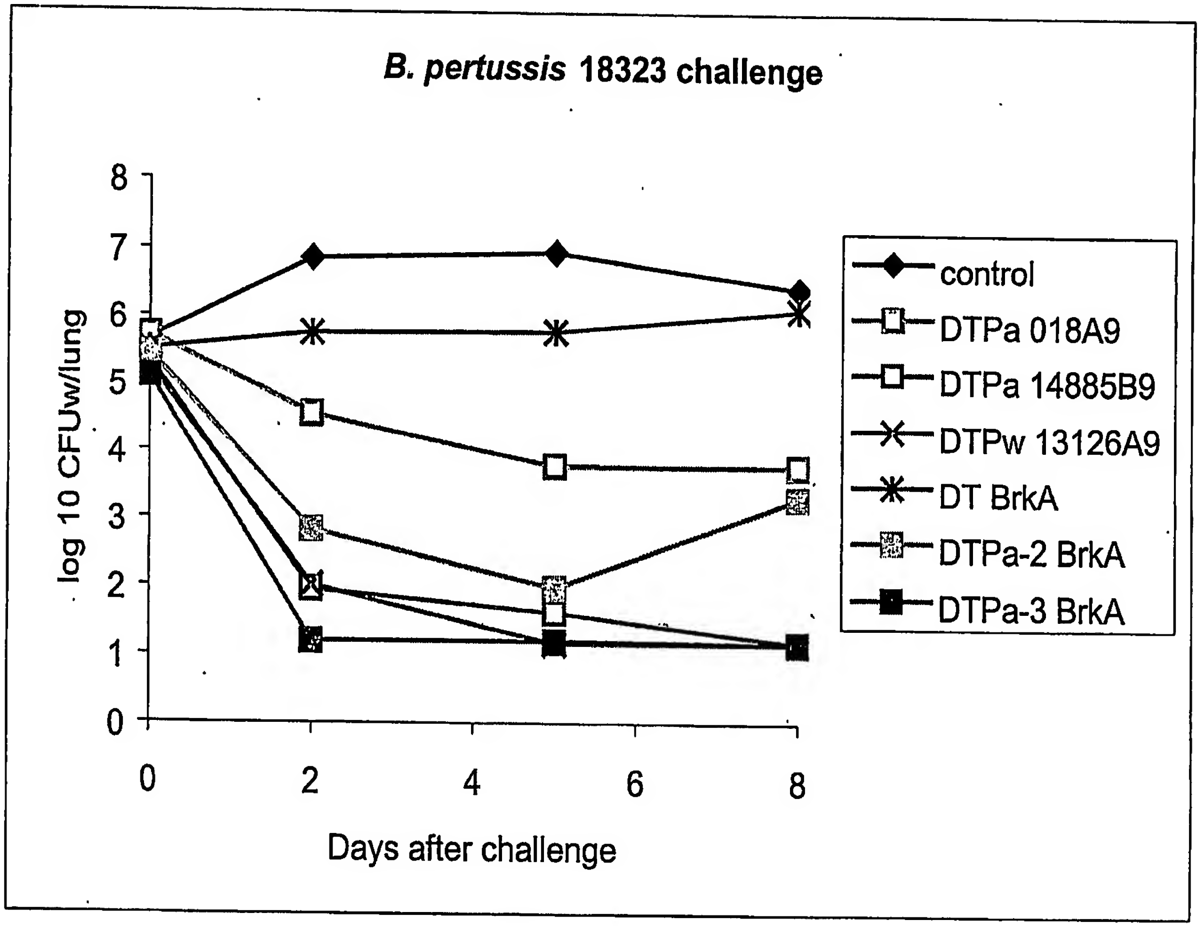
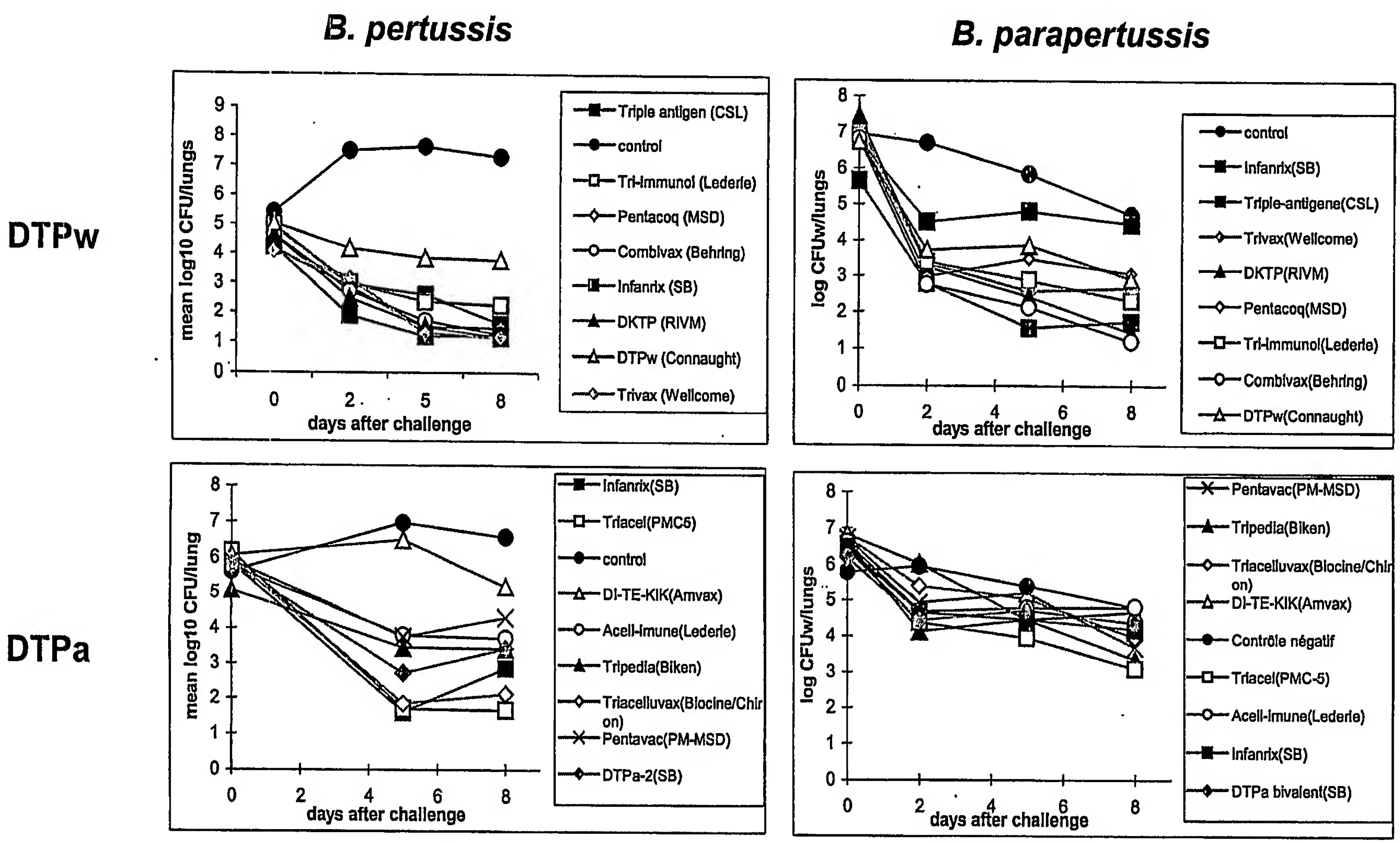

B. pertussis 18323 challenge
log 10 CFUw/lung
8
7
6
5
4
3
2
1
0
0
2
4
6
8
Days after challenge
control
DTPa 018A9
DTPa 14885B9
DTPw 13126A9
DT BrkA
DTPa-2 BrkA
DTPa-3 BrkA

Figure 3


B. pertussis
B. parapertussis
DTPw
DTPa
mean log10 CFU/lungs
days after challenge
Triple antigen (CSL)
control
Tri-Immunol (Lederle)
Pentacoq (MSD)
Combivax (Behring)
Infanrix (SB)
DKTP (RIVM)
DTPw (Connaught)
Trivax (Wellcome)
log CFUw/lungs
control
Infanrix(SB)
Triple-antigene(CSL)
Trivax(Wellcome)
DKTP(RIVM)
Pentacoq(MSD)
Tri-Immunol(Lederle)
Combivax(Behring)
DTPw(Connaught)
mean log10 CFU/lung
Infanrix(SB)
Triacel(PMC5)
control
DI-TE-KIK(Amvax)
Acell-Imune(Lederle)
Tripedia(Biken)
Triacelluvax(Biocine/Chiron)
Pentavac(PM-MSD)
DTPa-2(SB)
log CFUw/lungs
Pentavac(PM-MSD)
Tripedia(Biken)
Triacelluvax(Biocine/Chiron)
DI-TE-KIK(Amvax)
Contrôle négatif
Triacel(PMC-5)
Acell-Imune(Lederle)
Infanrix(SB)
DTPa bivalent(SB)

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